

Superoxide Dismutases in *Candida albicans*: Transcriptional Regulation and Functional Characterization of the Hyphal-induced *SOD5* Gene

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Superoxide dismutases (SOD) convert superoxide radicals into less damaging hydrogen peroxide. The opportunistic human pathogen *Candida albicans* is known to express CuZnSOD (*SOD1*) and MnSOD (*SOD3*) in the cytosol and MnSOD (*SOD2*) in the mitochondria. We identified three additional CuZn-containing superoxide dismutases, *SOD4*, *SOD5*, and *SOD6*, within the sequence of the *C. albicans* genome. The transcription of *SOD5* was up-regulated during the yeast to hyphal transition of *C. albicans*, and *SOD5* was induced when *C. albicans* cells were challenged with osmotic or with oxidative stresses. *SOD5* transcription was also increased when cells were grown on nonfermentable substrates as the only carbon source. The Rim101p transcription factor was required for all inductions observed, whereas the Efg1p transcription factor was specifically needed for serum-modulated expression. Deletion of *SOD5* produced a viable mutant strain that showed sensitivity to hydrogen peroxide when cells were grown in nutrient-limited conditions. Sod5p was found to be necessary for the virulence of *C. albicans* in a mouse model of infection. However, the *sod5* mutant strain showed the same resistance to macrophage attack as its parental strain, suggesting that the loss of virulence is not due to an increased sensitivity to macrophage attack.

INTRODUCTION

Aerobic eukaryotic pathogens can encounter superoxide radicals (O_2^-) generated from several sources. These sources can be internal or external. An important internal source is the mitochondrial respiratory chain (Boveris, 1978; Casteilla *et al.*, 2001; Lenaz, 2001), and thus the rate of respiration can have a significant impact on reactive oxygen species (ROS) production. A key external source of ROS encountered by pathogens is from phagocytes. The superoxide radical is the first intermediate in the oxidative burst generated in the phagosome, and this burst is thought to be involved in pathogen killing (Reeves *et al.*, 2002). The superoxide radicals are known to inactivate [4Fe-4S] cluster-containing enzymes by oxidizing one iron and releasing it from the cluster (Liochev and Fridovich, 1994; Fridovich, 1995). Free iron can react with hydrogen peroxide to generate toxic hydroxyl radicals (OH^\cdot) by Fenton chemistry (Fridovich, 1978; Meneghini, 1997). The hydroxyl and superoxide radicals react with cellular components, resulting in oxidation of proteins and nucleic acids as well as lipid peroxidation. These effects can lead to inactivation of enzymes, to disruption of membranes, to mutations, and ultimately to cell death (Halliwell and Gutteridge, 1990, 1999).

To reduce the harmful effects of superoxide radicals, cells express detoxifying enzymes. Superoxide dismutase (SOD) is an antioxidant enzyme involved in elimination of superoxide anions; it catalyzes the reaction: $O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$. Normally, H_2O_2 is still toxic to the cell; therefore, another enzyme, catalase, converts it to water. Superoxide dismutases can be classified according to metal cofactor(s) bound to them: there are iron (FeSOD), manganese (MnSOD), copper and zinc (CuZnSOD), and nickel (NiSOD) versions of the enzymes. The CuZnSOD family is thought to have evolved separately, and thus these enzymes share no sequence similarity with other SOD families (Kanematsu and Asada, 1991; Smith and Doolittle, 1992).

An important aerobic eukaryotic pathogen, *Candida albicans*, causes the majority of human fungal infections. These infections range from thrush in immunocompetent colonized hosts, to life-threatening systemic infections in immunocompromised individuals such as patients with cancer (Corner and Magee, 1997). *C. albicans* can grow in several morphological forms: it can proliferate as a budding yeast, and it can also form filaments, such as true hyphae and pseudohyphae. Hyphal formation of this fungus can be induced by high temperature, basic pH, and by the presence of serum, and this morphological transition is implicated in pathogenesis of *C. albicans* (Brown and Gow, 1999).

Typically, eukaryotes express cytosolic CuZnSOD and mitochondrial MnSOD (Fridovich, 1995). *C. albicans* possesses both cytosolic CuZnSOD and mitochondrial MnSOD encoded by the *SOD1* and *SOD2* genes, respectively (Hwang *et al.*, 1999; Rhie *et al.*, 1999). In addition to these proteins, a *C. albicans* cytoplasmic MnSOD (the *SOD3* gene product) was

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Table 1. Strains used in this study

Strain	Genotype	Source
SC5314	Wild-type isolate	Fonzi and Irwin (1993)
RM1000	URA3Δ::λimm434/URA3Δ::λimm434 HIS1::hisG/HIS1::hisG	Negredo <i>et al.</i> (1997)
RM1000-HU	URA3Δ::λimm434/URA3Δ::λimm434 HIS1::hisG/HIS1::hisG SOD5/SOD5::[p37H]::[p37U]	This study
H10	URA3Δ::λimm434/URA3Δ::λimm434 HIS1::hisG/HIS1::hisG sod5Δ::HIS1/SOD5	This study
UH16	URA3Δ::λimm434/URA3Δ::λimm434 HIS1::hisG/HIS1::hisG sod5Δ::hisG-URA3-hisG/sod5Δ::HIS1	This study
UH16-1	URA3Δ::λimm434/URA3Δ::λimm434 HIS1::hisG/HIS1::hisG sod5Δ::hisG/sod5Δ::HIS1	This study
UH16-2	URA3Δ::λimm434/URA3Δ::λimm434 HIS1::hisG/HIS1::hisG sod5Δ::hisG/sod5Δ::HIS1::[p37U]	This study
UH16-3	URA3Δ::λimm434/URA3Δ::λimm434 HIS1::hisG/HIS1::hisG sod5Δ::HIS1/ sod5Δ::hisG::[pSOD5-110]	This study
HLC52	URA3Δ::λimm434/URA3Δ::λimm434 efg1Δ::hisG/efg1Δ::hisG-URA3-hisG	Lo <i>et al.</i> (1997)
JKC19	URA3Δ::λimm434/URA3Δ::λimm434 cph1Δ::hisG/cph1Δ::hisG-URA3-hisG	Liu <i>et al.</i> (1994)
DAY25	URA3Δ::λimm434/URA3Δ::λimm434 HIS1::hisG/HIS1::hisG arg4::hisG/arg4::hisG rim101: ARG4/rim101::URA3	Davis <i>et al.</i> (2000a)
DAY185	URA3Δ::λimm434/URA3Δ::λimm434 HIS1::hisG/HIS1::hisG ARG4::URA3::arg4::hisG/arg4::hisG	Davis <i>et al.</i> (2000a)
BWP17	URA3Δ::λimm434/URA3Δ::λimm434 HIS1::hisG/HIS1::hisG arg4::hisG/arg4::hisG	Wilson <i>et al.</i> (1999)
GKO6	URA3Δ::λimm434/URA3Δ::λimm434 HIS1::hisG/HIS1::hisG arg4::hisG/arg4::hisG mds3::Tn7-UAU1/mds3::Tn7-URA3	Davis <i>et al.</i> (2002)

recently identified (Lamarre *et al.*, 2001). *SOD1* was shown to form homodimers (Lamarre *et al.*, 2001), whereas *SOD2* and *SOD3* were shown to form homotetramers (Rhie *et al.*, 1999; Lamarre *et al.*, 2001). *C. albicans* Sod1p was shown to protect cells against superoxide radicals produced by macrophage, and it was shown to be important for the virulence of *C. albicans* in mouse model (Hwang *et al.*, 2002). *C. albicans* catalase was also shown to be involved in virulence of this pathogen. *C. albicans* Sod2p was shown to have no effect on the virulence aspect of this fungus but rather protects the cell against intracellularly produced superoxides (Hwang *et al.*, 2003). *C. albicans* seems to coordinate the production of CuZnSOD and MnSOD in a reciprocal manner: CuZnSOD shows the highest activity in the midexponential phase, whereas MnSOD has the highest activity upon the entry to stationary phase (Rhie *et al.*, 1999; Lamarre *et al.*, 2001). The morphology of *C. albicans* is coupled to CuZnSOD production because cells generate increased amounts of CuZnSOD during the switch from yeast to hyphal forms (Gunasekaran *et al.*, 1998). In particular, the conditions of elevated production of CuZnSOD coincide with increased levels of ROS produced by *C. albicans* as cells produce the highest levels of ROS, including superoxide, during hyphal growth and in exponential phase (Schroter *et al.*, 2000). In addition, *C. albicans* generates higher amounts of ROS when grown in the presence of salt (Schroter *et al.*, 2000; Sander *et al.*, 2002).

We have identified three new potential *C. albicans* superoxide dismutase genes. We studied the expression, regulation, and function of one of these genes, *SOD5*, and show that this gene is under a complex regulation of expression. Functional tests show no involvement of *SOD5* in defense against macrophage-induced killing. However, the effects of the deletion of *SOD5* on virulence may suggest the involvement of Sod5p in removal of superoxides produced by the host.

MATERIALS AND METHODS

C. albicans and Bacterial Strains

The *C. albicans* strains are listed in Table 1; plasmids and oligonucleotides used in this study are listed in Table 2. The *C. albicans* strains SC5314 (Fonzi and Irwin, 1993), HLC52 (Lo *et al.*, 1997), JKC19 (Liu *et al.*, 1994), DAY25 and

DAY185 (Davis *et al.*, 2000a), BWP17 (Wilson *et al.*, 1999), and GKO6 (Davis *et al.*, 2002) were used for transcription analysis; RM1000 (Negredo *et al.*, 1997) was used as a parental strain to generate the RM1000-HU, H10, UH16, UH16-1, UH16-2, and UH16-3 strains. The *Escherichia coli* strain DH5α was used for all plasmid constructions and maintenance. The plasmid pVEC (Magee and Magee, 1997) was used for the generation of plasmids p37, p37H, p37U, and pSOD5-110; plasmids p5921 (Fonzi and Irwin, 1993) and pRM100 (Pla *et al.*, 1995) were used for the amplification of *hisG*-*CaURA3*-*hisG* and *CaHIS1* markers, respectively.

Media and Culture Growth Conditions

C. albicans strains were routinely cultured on YPD (1% yeast extract, 2% peptone, 2% dextrose, pH 6) medium at 30°C. Cells with integrated selection markers or disrupted genes were cultured in synthetic glucose medium containing 0.67% yeast nitrogen base, 0.15% amino acids, 2% dextrose, and appropriate supplements. All media were supplemented with uridine (25 μg/ml) for the growth of *C. albicans* Ura auxotrophic strains.

Because under certain conditions, *C. albicans* cells form hyphae at 37°C, it is difficult to accurately measure the growth of cells in the culture media. We therefore prepared a standard growth-curves YPD media at 37°C. We observed that it takes 300 min for SC5314 strain to reach an OD₆₀₀ of 1.0 at 30°C, and it takes 225 min for SC5314 strain to reach an OD₆₀₀ of 1.0 at 37°C. It takes 325 min for RM1000-HU and UH16-2 strains to reach an OD₆₀₀ of 1.0 at 30°C, and it takes 250 min for RM1000-HU and UH16-2 strains to reach an OD₆₀₀ of 1.0 at 37°C. These standard curves were used to approximate the time when the conditions should have changed.

For the Northern analyses performed with the yeast and mycelial forms of *C. albicans*, blastospores were first grown overnight at 30°C in the following media: mammalian cell culture media IMDM (pH 6.5) (Invitrogen, Carlsbad, CA), YPD, YP, or YP supplemented with either 2% galactose (YPGal), 3% glycerol (YPGlyc), or 1.5% methanol (YPMeth). The precultures were then diluted to an OD₆₀₀ of 0.1 and incubated in the same media at either 30°C until the OD₆₀₀ reached 1.0, or at 37°C until the OD₆₀₀ reached 2.5. In other cases, the preculture grown in YPD medium was diluted to an OD₆₀₀ of 0.1 and then incubated until the OD₆₀₀ reached 1.0 at either 30 or 37°C in the following media: YPD, YPD supplemented with 10% serum, YPD adjusted to pH 8, YPD with 0.3 M NaCl, and YPD with 0.3 M NaCl at pH 8.

For some samples, the preculture grown in YPD medium was diluted to an OD₆₀₀ of 0.1 and then incubated at either 30 or 37°C in YPD. In those cultures the pH, the NaCl concentration or both of those factors were increased 10, 30, or 60 min before the OD₆₀₀ reached 1.0. In those conditions, the pH of the media was increased to 8.0 with 10 N NaOH; the salt concentration was increased to 0.3 M by adding appropriate amount of 5 M NaCl solution into the media.

In other cases, the preculture grown in YPD medium was diluted to an OD₆₀₀ of 0.1. The culture was then supplemented with reactive oxygen species generators 1 h before the OD₆₀₀ reached 1.0 at 30°C, or 1 h before the OD₆₀₀ reached 2.5 at 37°C. In this case, cells were grown in YPD with 0.4 mM H₂O₂, YPD with 0.5 mM menadione (Sigma-Aldrich, St. Louis, MO), or YPD with 0.5 mM riboflavin (Sigma-Aldrich) (illuminated with 100-W table-lamp). All liquid cultures were rotated at 250 rotations per minute. Spot-assays were

Table 2. Plasmids and oligos used in this study

A. Plasmids	Description	Source
pVEC	<i>CaARS CaURA3</i>	Magee and Magee (1997)
p37	<i>CaARS</i> upstream flank 1kb <i>CaSOD5</i> downstream flank 1kb	This study
p37H	<i>CaARS</i> upstream flank 1kb <i>CaHIS1</i> downstream flank 1kb	This study
p37U	<i>CaARS</i> upstream flank 1kb <i>hisG-CaURA3-hisG</i> downstream flank 1 kb	This study
pSOD5-110	<i>CaARS CaURA3</i> upstream flank 1kb <i>CaSOD5</i> downstream flank 1kb	This study
p5921	<i>hisG-CaURA3-hisG</i>	Fonzi and Irwin (1993)
pRM100	<i>CaARS CaHIS1</i>	Pla <i>et al.</i> (1995)
B. Oligos	Sequence	Used for
ODH157(<i>EcoRI</i>)	GGAATTCAAACTGGTCTACCAGAAGTT	<i>SOD5</i> + 1kb flank F
ODH158(<i>EcoRI</i>)	GGAATTCGGTTATGTTAACGGTTAGAG	<i>SOD5</i> + 1kb flank R
OMM1(<i>Bam</i> HI)	CGGGATCCGATGAATGGTAAAGTTAGATTGATAT	Diverge <i>SOD5</i> F
OMM2(<i>Bam</i> HI)	CGGGATCCGAAAAATAAAATAGATGAGCCATT	Diverge <i>SOD5</i> R
OMM3(<i>Xba</i> I)	GCTCTAGAAAAGAAAAATAAAATAGATGAGCC	Diverge <i>SOD5</i> R
OMM4(<i>Xba</i> I)	GCTCTAGAGGTACCTGGAGGATGAGGAG	<i>CaHIS1</i> F
CB20R(<i>Bam</i> HI)	CGGGATCCCAATATTTATGAGAACTATCACTTC	<i>CaHIS1</i> R
OMM8	GGAGCAGTAGAAGCCATACTA	<i>SOD5</i> R Northern
OMM9	ATGGGCGAGTCTTACAAAAC	<i>SOD5</i> F Northern
OMM10	ACCAGTGAATCATTTGAAGTT	<i>SOD4</i> F Northern
OMM11	AGAAGCACTAGTTGATGAACC	<i>SOD4</i> R Northern
OMM14	TCCAGAAGATGATGAAAGACATG	<i>SOD1</i> F Northern
OMM15	GTCTAGCACCAGCATGACCAG	<i>SOD1</i> R Northern
OMM16	AACACAACCTTGCTTTGAGTTGAAA	<i>SOD6</i> F Northern
OMM17	ACAATCACTAGAGATTCGGTGGAT	<i>SOD6</i> R Northern
OMM18(<i>Bam</i> HI)	CGGGATCCCGGTTATGTTAACGGTTAGAG	<i>SOD5</i> + 1kb flank R
OMM19(<i>Xba</i> I)	GCTCTAGAAAACCTGGTCTACCAGAAGTT	<i>SOD5</i> + 1kb flank F
UO106	AAGCCGGTTTTGCCGGTG	<i>ACT1</i> probe F
UO107	TGGTGAACAATGGATGGAC	<i>ACT1</i> probe R

done on media solidified with 2% agar. 5'-Fluoroorotic acid (Diagnostic Chemicals, Charlottetown, PEI, Canada) containing medium was prepared as described previously (Boeke *et al.*, 1984), except that uridine was substituted for uracil. *C. albicans* cells were transformed by the previously described method (Chen *et al.*, 1992).

RNA Preparation and Northern Blot Analyses

Total RNA was extracted by the hot phenol extraction method (Carlson and Botstein, 1982). The Northern blot analyses were performed by the previously described method (Alarco *et al.*, 1997) with the following modifications. RNA samples (80 µg) were electrophoresed on a 7.5% formaldehyde, 1% agarose gel and transferred by capillarity to a Zeta-Probe nylon membrane (Bio-Rad, Mississauga, ON, Canada). Detection of specific RNAs was performed by hybridization at 65°C in 0.5 M NaPO₄, pH 7.2, 1 mM EDTA, 7% SDS, 1% bovine serum albumin with 50 ng of ³²P-labeled DNA probes (single-stranded DNA was omitted from this solution). Probes were generated by using the random prime labeling system (Amersham Biosciences, Piscataway, NJ) with the following modifications: 50 ng (rather than 25 ng) of DNA was added to the reaction, the reaction was incubated at 37°C for 45 min (rather than for 10 min), and all of labeled probe (rather than 1.25 ng) was used for hybridization. The unincorporated labeled nucleotides were removed by G-50 microcolumns (Amersham Biosciences). Membranes were washed once at 65°C with a solution containing 40 mM NaPO₄, pH 7.2, 5% SDS, 1 mM EDTA, 0.5% bovine serum albumin for 10 min (rather than twice for 20 min), and once with a solution containing 40 mM NaPO₄, pH 7.2, 1% SDS, and 1 mM EDTA for 20 min (rather than four times for 20 min) and were visualized using a PhosphorImager (model 4255; PerkinElmer Life Sciences, Boston, MA).

All Northern probes were made by polymerase chain reaction (PCR): the 0.24-kb-long *SOD5* probe used primers OMM8 and OMM9, the 0.24-kb-long *SOD4* probe used primers OMM10 and OMM11, the 0.21-kb-long *SOD1* probe used primers OMM14 and OMM15, and the 0.5-kb-long *SOD6* probe used primers OMM16 and OMM17. A *CaACT1* probe was generated with UO106 and UO107 oligos and was used as an internal control to monitor RNA loading and transfer (a gift from Dr. Ursula Oberholzer, Biotechnology Research Institute, Montreal, PQ, Canada).

The dot-blot was performed the same way as the Northern, with the difference that 80 µg of *SOD1*, *SOD4*, *SOD5*, and *SOD6* unlabeled probes (rather than 80 µg of RNA) were spotted on the membranes.

Deletion and Reversion of *SOD5*

To delete the *SOD5* gene, the *SOD5* open reading frame (ORF) and its 1-kb flanking regions were PCR amplified from genomic DNA of the CAI4 strain

with oligos ODH157 and ODH158 that contained *EcoRI* sites. The PCR product (2.6 kb) and plasmid pVEC were digested with *EcoRI*, and a 4.7-kb *EcoRI* fragment of pVEC was gel-purified and ligated with the *EcoRI*-digested 2.6-kb *SOD5* PCR product. Clone p37 contained an insert with the *SOD5* ORF running in the opposite orientation with respect to that of the Amp marker. The *SOD5* ORF was deleted from p37 by the use of inverse PCR by using either oligonucleotides OMM1 and OMM2 or OMM1 and OMM3. The *hisG-URA3-hisG* marker was cut from p5921 with *Bgl*II and *Bam*HI; OMM1 and OMM2 create *Bam*HI restriction sites in p37 into which the *hisG-URA3-hisG* marker was inserted to yield p37U. The *HIS1* marker was PCR amplified from pRM100 with OMM4 (containing an *Xba*I site) and CB20R (containing a *Bam*HI site, a gift from Dr. Catherine Bachewich, Biotechnology Research Institute, Montreal, PQ, Canada); OMM1 and OMM3 created *Bam*HI and *Xba*I restriction sites, respectively, in p37 into which the *HIS1* marker was ligated to yield p37H. The *HIS1* and *SOD5*-1-kb-flanking regions were PCR amplified from p37H with oligos ODH157 and ODH158, and this PCR product was transformed into RM1000 to yield the H10 strain, in which the *sod5::HIS1* fragment replaces the chromosomal *SOD5* ORF. The *hisG-URA3-hisG* and *SOD5*-1-kb-flanking regions were cut out of p37U with *Xmn*I and *Spe*I, and this fragment was transformed into H10 to yield the UH16 strain, in which the *sod5::hisG-URA3-hisG* fragment replaces the second chromosomal *SOD5* ORF. Strain UH16 was plated on 5'-fluoroorotic acid-containing plates to select for Ura-auxotrophs. As a result, the UH16-1 strain was created in which one *SOD5* ORF was replaced with *HIS1* marker, and the second *SOD5* ORF was replaced with *hisG*. To create the prototrophic strain UH16-2, UH16-1 was transformed with the p37U construct, which was targeted to the chromosomal *SOD5*-promoter region by cutting the construct in the *SOD5*-promoter region with *Nsi*I. The *HIS1 URA3* auxotrophic wild-type strain was transformed with the p37U and p37H constructs in a sequential manner; these were targeted to chromosomal *SOD5*-promoter region by cutting the constructs in the *SOD5*-promoter region with *Nsi*I, creating strain RM1000-HU. For *SOD5* reversion studies, the *SOD5* ORF and its 1-kb-flanking regions were PCR amplified from CAI4 genomic DNA with the OMM18 and OMM19 oligos that contained *Bam*HI and *Xba*I sites, respectively. The resulting PCR product (2.6 kb) and plasmid pVEC were double digested with *Bam*HI and *Xba*I restriction enzymes and ligated together to yield pSOD5-110. pSOD5-110 was transformed into UH16-1 to yield revertant strain UH16-3.

Genomic DNA was prepared from all of the obtained strains, digested with *Nhe*I and *Xho*I, and analyzed for the correct integration events by Southern blotting with a digoxigenin system (Roche Diagnostics, Indianapolis, IN). The digoxigenin probe was generated by PCR with ODH157 and OMM1 oligos that amplified the 1-kb *SOD5* promoter sequence from p37. To confirm the

absence of the *SOD5* ORF, genomic DNA from UH16, UH16-1, and UH16-2 strains were cut with *DraI*, and analyzed by Southern blotting with a probe that binds inside the *SOD5* ORF. This probe was also used for Northern analysis to confirm the absence of *SOD5* mRNA in the *SOD5* inducing conditions (see MATERIALS AND METHODS).

Protein Preparation and In-Gel SOD Activity Assay

Protein preparation was done as described previously (Ito-Kuwa *et al.*, 1999). The protein concentration was determined by the Bradford method using an assay kit (Bio-Rad), as described by the provider. SOD activity in polyacrylamide gels was detected using nitroblue tetrazolium (Beauchamp and Fridovich, 1971), with the modification that 5 mg of cell extracts was electrophoresed on 10% nondenaturing polyacrylamide gels in triplicates. After the electrophoresis, one gel was used for Coomassie Blue staining (Malloy *et al.*, 1984). The other two gels were used for determining SOD activity with or without 4 mM KCN (Ito-Kuwa *et al.*, 1999).

Spot Assay and Growth Assay

Overnight, cultures of *C. albicans* cells were concentrated to OD₆₀₀ of 20.0. Serial twofold dilutions were done, and 5 μ l of cells was spotted onto a plate and incubated for 48 h at either 30 or 37°C. Spot assays were done on the types of media described in RESULTS. The growth assay of cells in liquid media was done by diluting overnight cells in those media to OD₆₀₀ of 0.1. The growth rate was monitored at OD₆₀₀ for 24 h at 30 and 37°C.

Macrophage and Virulence Assays and Fungal Burden

The end point dilution survival assay was performed as described previously (Marcil *et al.*, 2002). Dilutions of *C. albicans* without macrophages where colonies could be distinctively visualized were counted toward the lower limit and compared with the same dilutions with macrophages. Colonies from a total of at least 72 wells per condition were used to provide data.

For virulence assays, *C. albicans* strains were passaged overnight at 30°C in YPD before infection. Cells were harvested, washed in cold phosphate-buffered saline (Wisent Inc., St-Bruno, Canada), and counted using a hemacytometer. Then cells were resuspended at a density of 5.75×10^6 cells/ml. B6 mice (8-wk-old females) (Charles River Laboratories, Wilmington, MA) were infected through the lateral tail vein with 200 μ l (1.15×10^6 organisms). Mouse survival was monitored at least three times daily, and moribund mice were killed by CO₂ gas. We used *C. albicans* strain RM1000-HU to infect 13 mice, and UH16-2 and UH16-3 to infect 12 mice. *C. albicans* colony-forming units (cfu) in kidney were counted from live animals killed at day 5 after the infection. The kidneys were removed aseptically from four mice per strain; the organs were weighed, homogenized, diluted in sterile saline, and plated on YPD plates. Colonies were counted after incubation of the plates at 30°C for 48 h, and the results were expressed as cfu per gram of infected organ.

RESULTS

Identification of *SOD4*, *SOD5*, and *SOD6*

The mammalian pathogen *C. albicans* is known to possess both a copper-zinc-dependent superoxide dismutase (CuZnSOD encoded by *SOD1*) (Hwang *et al.*, 1999) and a manganese-dependent superoxide dismutase (MnSOD, encoded by *SOD3*) (Lamarre *et al.*, 2001) in the cytosol and to encode a second MnSOD (*SOD2*) that is localized to mitochondria (Rhie *et al.*, 1999). The *C. albicans* genome sequence revealed three additional open reading frames with high sequence homology to the copper-zinc family of superoxide dismutases: orf 19.2062, orf 19.2060, and orf 19.2108 in version 19 of Stanford's *C. albicans* genome sequence assembly (Stanford Genome Technology Center, <http://www-sequence.stanford.edu/group/candida/>). We have designated these new genes as *SOD4*, *SOD5*, and *SOD6* respectively. All three of these genes share sequence homology to the previously described *SOD1* and to each other, but share no homology to the manganese-containing superoxide dismutases (Figure 1A). All three predicted proteins (Sod4p, Sod5p, and Sod6p) contain characteristic residues involved in copper and zinc binding, and amino acids involved in dimer contact (Fink and Scandalios, 2002) (Figure 1B). Northern analysis (described below) showed that the mRNAs for each of the three genes were ~700 nucleotides in length. However, the predicted length of Sod6p of 316 amino acids was too large to be easily accommodated within

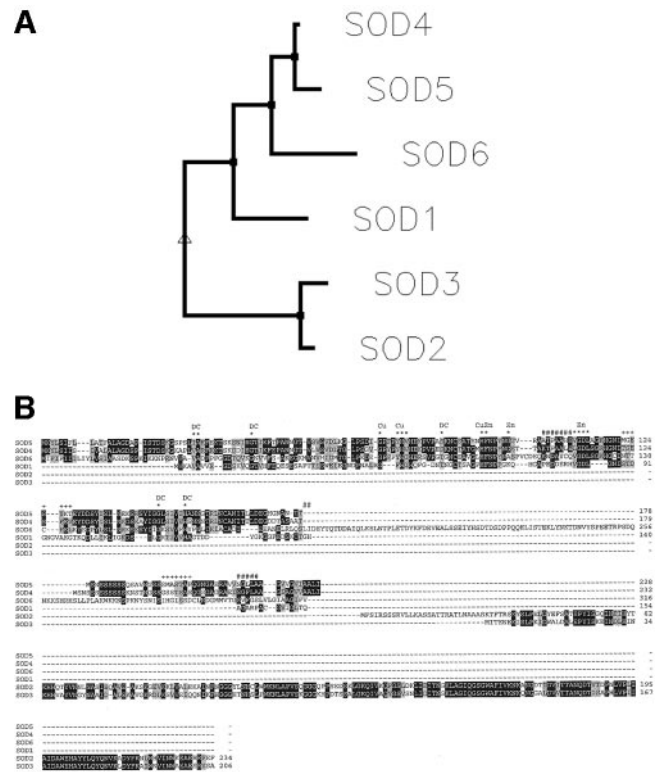
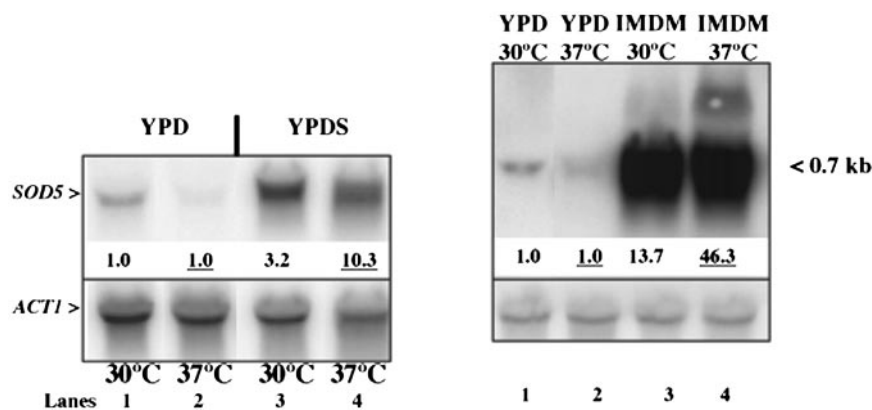


Figure 1. Similarity of the CuZn-containing and Mn-containing superoxide dismutases in *C. albicans*. (A) Phylogenetic tree shows the similarity between all *C. albicans*' superoxide dismutases: Sod4p, Sod5p, and Sod6p are homologous to Sod1p, and the CuZnSODs share no sequence similarity with the MnSODs. The analysis was produced by the Multiple Sequence Alignment program of Florence Corpet (<http://prodes.toulouse.inra.fr/multalin/multalin.html>). (B) Amino acid sequence alignment of CuZn Superoxide dismutases (*SOD1*, *SOD4*, *SOD5*, and *SOD6*) and Mn superoxide dismutases (*SOD2* and *SOD3*). Deduced amino acid sequences were aligned with the CLUSTALW program. Identical amino acids are boxed in black, and conservative changes are boxed in gray. Hyphens indicate gaps that were introduced to maximize the alignment. +, oligos used for Northern and Southern analysis for Sod4p, Sod5p, and Sod6p. #, oligos used for Northern and Southern analysis for Sod1p. The Sod4p, Sod5p, and Sod6p contain CuZn-SOD characteristic residues. Asterisks below Cu and Zn stand for copper and zinc metals bindings, respectively; asterisks below DC stand for amino acids involved in dimer contact (Fink and Scandalios, 2002). Numbers at the ends of the sequences represent the amino acid lengths of the proteins.

this size of message. This discrepancy could be resolved if *SOD6* contained an intron. Nucleotide sequence analysis shows that *SOD6* contains a well-characterized 3' splicing site "CTAGG" motif starting at nucleotide 752, a signal for lariat formation "AACTGAC" starting at nucleotide 723, and a possible 5' splicing site "GTCGGT" starting at nucleotide 462, which closely resembles the previously reported "GTANGT" motif (Ballance, 1986). Splicing of this potential intron would generate a predicted ORF of 218 amino acids, close in size to the predicted 232 amino acids and 228 amino acids of Sod4p and Sod5p. All these proteins are likely to be cytosolic, because they lack mitochondria-targeting sequences. Intriguingly, *SOD4* and *SOD5* are close together in the genome, with *SOD4* located 2 kb upstream of *SOD5* (Nantel *et al.*, 2002).



from *SOD5* intensities obtained at 30°C. Quantifications were performed by NIH Image software (RSB-NIMH-NIH, <http://rsb.info.nih.gov/nih-image/>).

SOD5 Is Transcriptionally Induced during Yeast to Hyphal Transitions

Hyphal formation in *C. albicans* can be induced by incubation of the cells in the presence of serum and high temperature. Recent transcriptional profiling experiments established that the *SOD5* gene was induced during the yeast to hyphal switch (Nantel *et al.*, 2002). To study the expression pattern of the copper-zinc SOD genes, we generated probes to unique sequences within each gene (Figure 1B). Dot-blot analysis confirmed the specificity of each probe; there was an insignificant cross-hybridization between the *SOD4* and *SOD5* probes, and no cross-hybridization for any of the other combinations (our unpublished data). These probes were used to investigate transcriptional regulation of the *SOD5* gene under a variety of conditions. Northern blot analysis of the actin (*ACT1*) mRNA in *C. albicans* strains was used as an internal loading control, because the transcription of this gene was shown to be unaffected under most of the growth conditions tested in our study (Nantel *et al.*, 2002). In agreement with microarray data, Northern analysis demonstrated that the *SOD5* transcription increases at 37°C in the presence of serum, compared with the control condition of no serum and 30°C (Figure 2A, lanes 1 and 4). We investigated whether serum, temperature or both of these factors regulated the expression of *SOD5*. In the absence of serum, a temperature increase from 30 to 37°C reduced the expression of *SOD5* from a relative intensity of 1.0–0.3 (Figure 2A, lanes 1 and 2). In contrast, the presence of serum caused high levels of *SOD5* expression (Figure 2A, compare line 1 with lines 3 and 4). This suggests that serum, rather than temperature, controls the induction of *SOD5* expression.

The mammalian cell culture medium IMDM lacks serum but allows *C. albicans* to grow exclusively in a hyphal form at both 30 and 37°C. *SOD5* was highly expressed in this medium at both temperatures (Figure 2B). As in Lamarre *et al.*, 2001, we observed the accumulation of new, 3.4-kb mRNA species hybridizing to the *SOD5* probe (lane 4), which does not correspond to the specific *SOD1*, *SOD4*, or *SOD6* mRNAs (our unpublished data). The nature of this mRNA is unclear, but because the distance between the start codon of *SOD5* and the stop codon of *SOD4* on the chromosome is 3.3 kb, it is possible that the *SOD5* probe hybridized to a mRNA species that contains both *SOD4* and *SOD5*. This idea is supported by the detection of the same message by a *SOD4* probe (our unpublished data).

Another way to induce the yeast-to-hyphal transition is to increase the pH and the temperature of the medium. At both

temperatures, 30 and 37°C, an increase of pH from 6.0–8.0 increases the expression of *SOD5* (Figure 3A, lanes 6–9 and 18–21). At both temperatures, the rise in *SOD5* mRNA expression persists up to 300 min for 30°C or 225 min for 37°C of growth in the media with an increased pH. The induction peaks at 30 min for cells grown at 30°C and at 10 min for cells grown at 37°C.

Osmotic and Oxidative Stresses Affect *SOD5* Transcription

C. albicans cells grown in the presence of salt were shown to generate higher amounts of ROS (Schroter *et al.*, 2000; Sander *et al.*, 2002). Intriguingly, previous transcription profiling established that osmotic stress also induced the expression of *SOD5* (Enjalbert *et al.*, 2003). We confirmed these results by Northern analysis, because cells grown in the presence of high salt concentrations showed an increased transcription of *SOD5* at 30°C (Figure 3A). The levels of *SOD5* mRNA increase as early as 10 and 30 min after the addition of salt to the media at both 30 and 37°C but stay up after 60 and 300 min only at 30°C (Figure 3A, lanes 1–5 and 13–17).

When pathogens are engulfed by neutrophils, there is an increase in both pH and salt concentration inside the phagosome (Reeves *et al.*, 2002). Intriguingly, high salt and high pH have a cumulative effect on *SOD5* transcription at 30°C; at 37°C high salt and high pH yield to a higher *SOD5* response compared with the levels of *SOD5* transcripts induced separately by high pH and high salt (Figure 3A, lanes 10–12 and 22–23). The fact that high salt and high pH together lead to increased *SOD5* expression was expected for a model that the expression increase permits the mobilization of superoxide dismutase activity to defend against phagosome-mediated killing.

In some organisms such as *S. cerevisiae*, the transcription of the gene encoding the cytoplasmic superoxide dismutase is induced by treatment with various oxidative agents like hydrogen peroxide and menadione (Yoo *et al.*, 1999; Lamarre *et al.*, 2001; Lee *et al.*, 2002). We investigated the transcription profile of the *C. albicans* CuZn superoxide dismutases in cells treated either with hydrogen peroxide or superoxide radicals. We used two types of superoxide generators: riboflavin, which generates superoxides outside of the cell (Ito-Kuwa *et al.*, 1999), and menadione, which creates superoxides inside the cell (Lee *et al.*, 2002). The presence of hydrogen peroxide caused increased levels of *SOD1* expression (Figure 3B, compare lane 1 with lanes 3 and 4) and

Figure 2. Hyphal-inducing conditions induce *SOD5* expression. (A) Serum, rather than temperature, induces *SOD5* transcription. Northern blot analysis of the *SOD5* transcripts in *C. albicans* strain SC5314 grown in the log phase in indicated media (YPD and YPD containing 10% serum) at indicated temperatures. (B) *SOD5* transcription is up-regulated in IMDM medium. Northern blot analysis of the *SOD5* transcripts in *C. albicans* strain SC5314 grown in the log phase in IMDM medium at 30 and 37°C. Northern blot analysis of the actin (*ACT1*) transcripts in *C. albicans* strain SC5314 was used as an internal loading control. Relative intensities are shown below each of the genes analyzed. Relative intensities of cells grown at 37°C are underlined and quantified independently

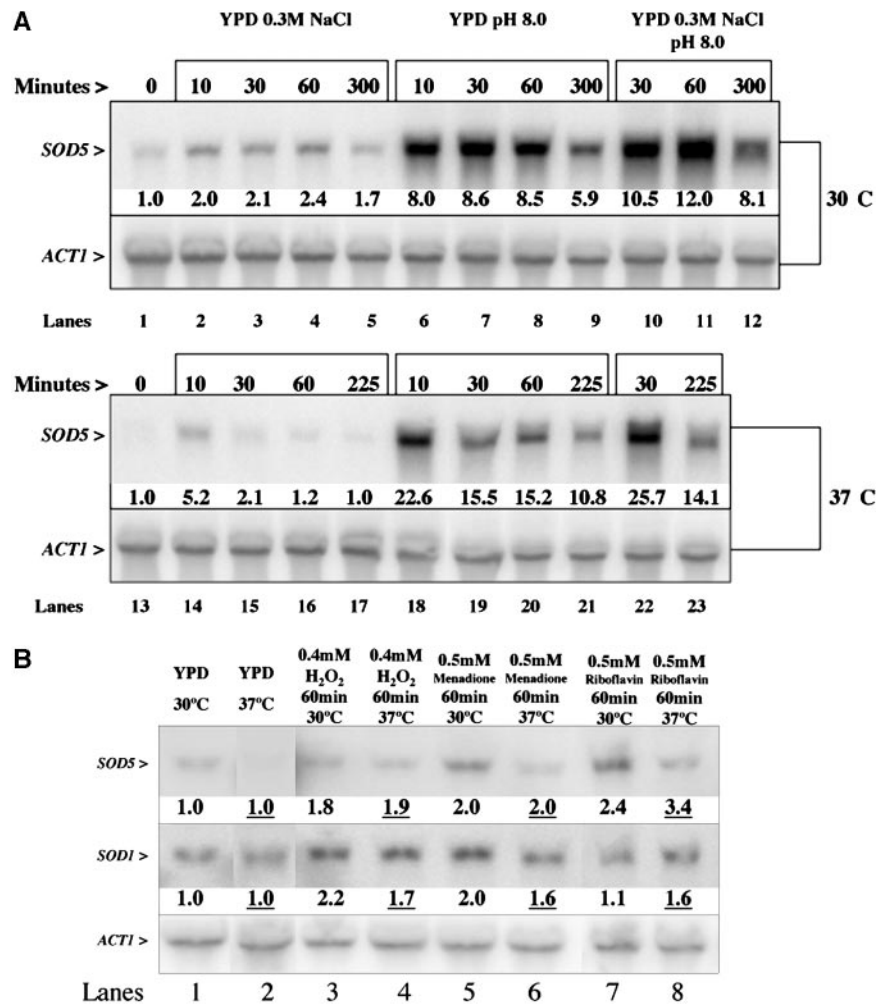


Figure 3. Alkaline conditions, osmotic and oxidative stresses affect *SOD5* transcription. (A) *SOD5* transcription is up-regulated in alkaline conditions and in the presence of increased amounts of salt. Northern blot analysis of the *SOD5* transcripts in *C. albicans* strain SC5314 diluted to an OD₆₀₀ of 0.1 and then incubated at either 30 or 37°C in YPD. In those cultures, the pH, NaCl concentration, or both of those factors were increased 10, 30, 60, or 300/225 min before the OD₆₀₀ reached 1.0. (B) Effects of oxidative shock on *SOD5* and *SOD1* of *C. albicans*. Northern blot analysis of the *SOD5* transcripts in *C. albicans* strain SC5314 grown in log-phase, from OD₆₀₀ of 0.1 to OD₆₀₀ of 1.0 at 30°C or from OD₆₀₀ of 0.1 to OD₆₀₀ of 2.5 at 37°C; the hydrogen peroxide/menadione/riboflavin were supplemented 1 h before reaching the final OD₆₀₀ of 1.0 or 2.5. Lanes 1 and 2, no oxidative shock was applied. Lanes 3 and 4, hydrogen peroxide was added 1 h before OD₆₀₀ reached 1.0. Lanes 5 and 6, menadione was added 1 h before OD₆₀₀ reached 1.0. Lanes 7 and 8, riboflavin and light were added 1 h before OD₆₀₀ reached 1.0. Northern blot analysis of the actin (*ACT1*) transcripts in *C. albicans* strain SC5314 was used as an internal loading control. Analysis as described in Figure 2.

moderate increases in transcription of *SOD5*. Menadione leads to moderate increases in transcription of *SOD1* at both temperatures and that of *SOD5* (Figure 3B, lanes 5 and 6). Riboflavin treatment results in elevated levels of *SOD1* at 37°C and that of *SOD5* at both temperatures (Figure 3B, lanes 7 and 8). The *SOD1* induction was weak, as described previously (Lamarre *et al.*, 2001). The fact that the transcription of *SOD5* was increased upon the induction of oxidative stress is also expected if the gene is involved in defense against a phagosome-generated oxidative burst. Because *SOD5* transcription was induced by both intracellular and extracellular superoxide generators, it provides us with the first evidence that Sod5p is needed for elimination of superoxides whose source is from within the cell.

Nonfermentable Carbon Sources Affect *SOD5* Expression

Because we saw that the intracellular superoxide generator menadione induced *SOD5* levels, we further investigated the effect of intracellularly generated superoxide on *SOD5* transcription. Studies of isolated mitochondria showed that up to 80% of the superoxide in eukaryotic cells was produced in the mitochondria by electron leakage at the QH2: cytochrome *c* segment (complex III) during respiration (Chance *et al.*, 1979). Therefore, the effect of respiration on *SOD5* transcription was investigated. One of the ways to increase respiration is to grow the cells in media with a nonfermentable carbon source, such as glycerol or methanol,

while providing glucose or galactose as the sole carbon source allows cells to grow by fermentation (Longo *et al.*, 1996; De Freitas *et al.*, 2000).

At 30°C, we grew cells until they reached an OD₆₀₀ of 1.0. We observed that the growth on glucose or galactose as the only carbon source leads to relatively low levels of *SOD5* transcription (Figure 4, lanes 1 and 3). When cells were grown in the presence of glycerol or methanol as the only carbon source at 30°C *SOD5* expression was moderately increased (Figure 4, lanes 5 and 7), compared with fermentable carbon sources at the same temperature (compare with lanes 1 and 3). At 30°C, the highest *SOD5* transcript levels were detected in cells grown in the absence of any carbon source (YP) (Figure 4, lane 9).

At 37°C, we grew cells until they reached an OD₆₀₀ of 2.5. We observed that the growth on glucose or galactose as the only carbon source leads to very low levels of *SOD5* transcription (Figure 4, lanes 2 and 4). When cells were grown in the presence of glycerol or methanol as the only carbon source at 37°C, *SOD5* expression was highly increased (Figure 4, lanes 6 and 8), compared with fermentable carbon sources at the same temperature (compare with lanes 2 and 4). At 37°C, the highest *SOD5* transcript levels were detected in cells grown in the absence of any carbon source (YP) (Figure 4, lane 10). These results show that growth on nonfermentable carbon sources increase *SOD5* transcription, which might suggest that one of the roles of Sod5p is to

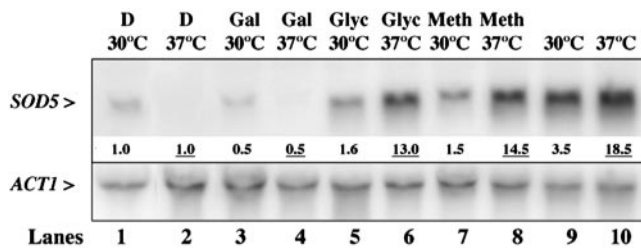


Figure 4. *SOD5* transcription is affected by nonfermentable carbon source. Northern blot analysis of the *SOD5* transcripts in *C. albicans* strain SC5314 grown in log-phase, from OD₆₀₀ of 0.1 to OD₆₀₀ of 1.0 at 30°C or from OD₆₀₀ of 0.1 to OD₆₀₀ of 2.5 at 37°C in YPD, YPGal, YPGlyc, YPMeth, and YP. Northern blot analysis of the actin (*ACT1*) transcripts in *C. albicans* strain SC5314 was used as an internal loading control. Analysis as described in Figure 2.

eliminate intracellularly generated superoxide radicals. *SOD1* and *SOD4* were also up-regulated by increased temperature when grown on nonfermentable carbon sources, and *SOD1*, *SOD4*, and *SOD6* were also up-regulated in the absence of carbon sources (YP) (our unpublished data).

Transcription Factors Involved in the Regulation of *SOD5* Expression

Previous transcription profiling established that the nonhyphal *efg1 cph1* double mutant failed to induce *SOD5* expression under hyphal-inducing conditions (Nantel *et al.*, 2002). We used Northern analysis to determine the relative impact of each transcription factor on the expression of the *SOD5* gene. For each of the individual null mutants, we attempted to induce *SOD5* transcription by one of the following conditions: serum and high temperature, osmotic stress (0.3 mM NaCl), and basic pH (pH8). In all of these conditions, the *cph1* mutant showed the same pattern of *SOD5* expression as the wild-type strain: *SOD5* was induced in all three conditions as compared with the noninduced condition (YPD 30°C) (Figure 5, lanes 1–8). On the other hand, the *efg1* mutant failed to increase the transcription of *SOD5* specifically under serum-inducing conditions (Figure 5, lanes 9–12, compare lanes 2 and 10). In addition, under noninducing conditions the *efg1* mutant showed generally elevated levels of *SOD5* message (Figure 5, compare lanes 1 and 9). Interestingly, the addition of salt to the media does not increase the *SOD5* mRNA levels, compared with *SOD5* levels during the growth of *efg1* mutant strain under noninducing conditions (Figure 5, lanes 9 and 11). The *SOD5* mRNA levels were increased in the *efg1* mutant in alkaline-inducing conditions, although not to the same extent as the corresponding increase in the wild-type strain (Figure 5, lane 12). To investigate the factors responsible for the pH-mediated induction of *SOD5* expression, we looked at cells defective in either the Rim101p transcription factor or in Mds3p; these factors represent independent branches of the pH response (Davis *et al.*, 2002). In contrast to both the *efg1* and *cph1* mutants, the *rim101* mutant blocked *SOD5* induction under all of the conditions tested compared with *SOD5* expression in its parental strain (Figure 5, lanes 13–20). Just as in the wild-type and the *cph1* mutant, the *mds3* mutant showed that *SOD5* was induced in all three conditions (YPD 30°C) (Figure 5, lanes 21–28). Therefore, the Cph1p transcription factor and Mds3p have essentially no effect on *SOD5* transcription under the conditions tested, Efg1p is required for *SOD5* transcription in the presence of serum and perhaps for suppressing the *SOD5* expression under the noninducing

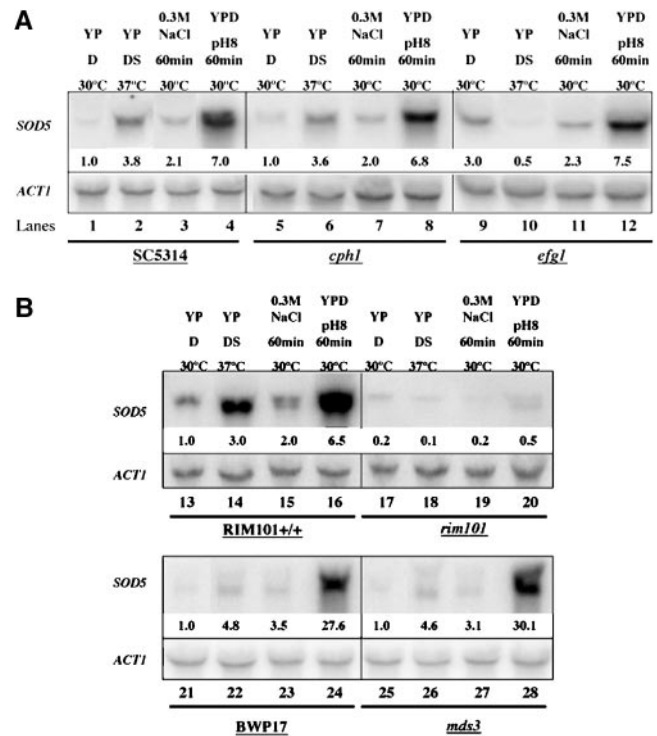
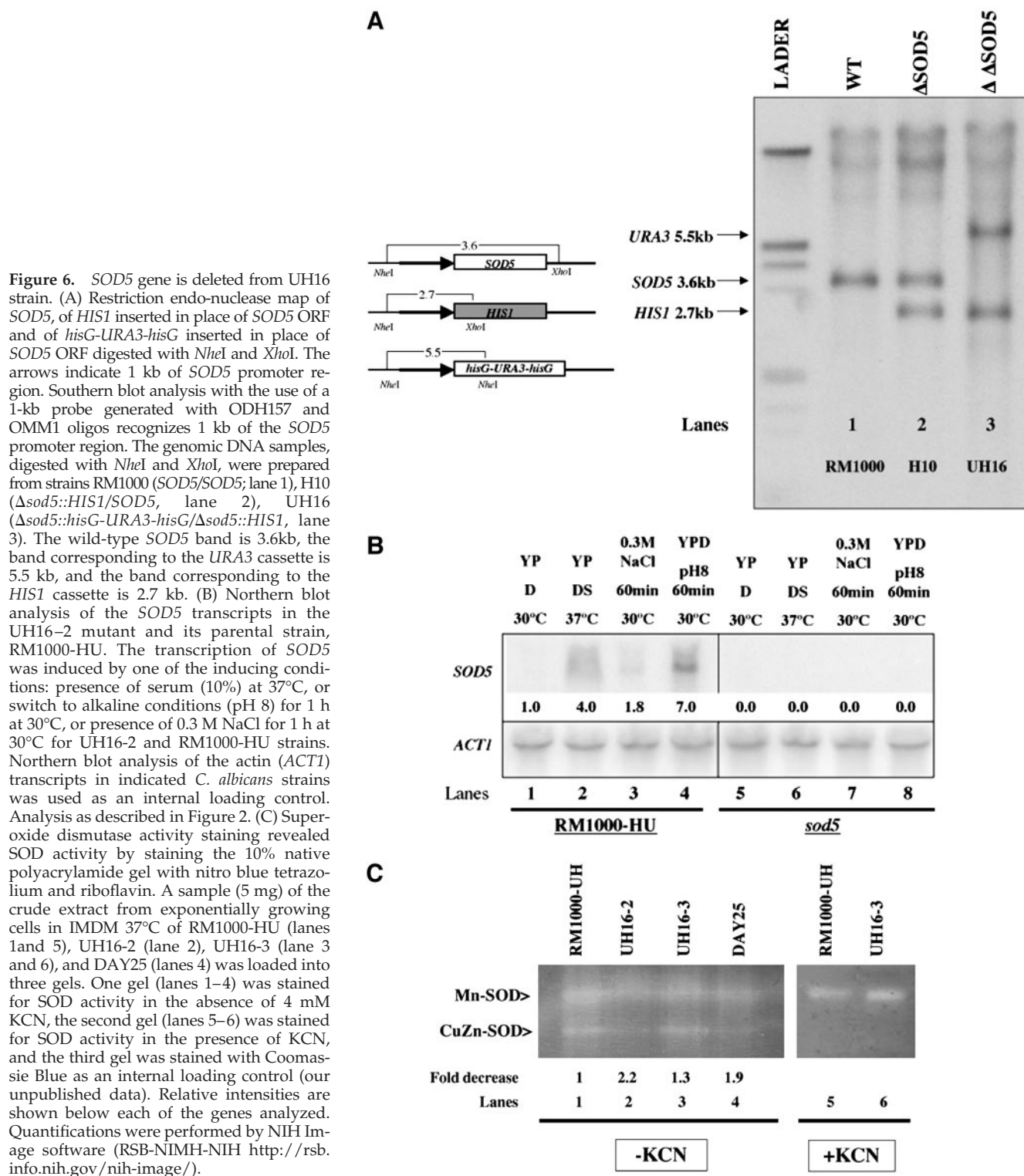


Figure 5. Transcription factors involved in the transcription of *SOD5*. Northern blot analysis of the *SOD5* transcripts in *C. albicans* strains lacking indicated transcription factors (strains SC5314, JKC19, HLC52, DAY25, DAY185, BWP17, and GKO6 [*mds3*]; Table 1). The potential transcription of *SOD5* was induced by one of the three inducing conditions: presence of serum (10%) at 37°C, presence of salt (0.3M NaCl) for 1 h at 30°C, or switch to alkaline conditions (pH8) for 1 h at 30°C. Analysis as described in Figure 2. The intensities of *SOD5* in HLC52 were compared with *SOD5* intensities of JKC19. The intensities of *SOD5* in DAY25 were compared with *SOD5* intensities of DAY185. The intensities of *SOD5* in *mds3* were compared with *SOD5* intensities of BWP17. These Northern blot analyses were reproduced twice. The general drop in *SOD5* levels in *rim101* was reproduced, but the difference in relative intensities of *SOD5* in the *rim101* strain was not significant.

condition, and Rim101p is important for the normal *SOD5* transcription under all of the conditions tested.

SOD5 Is Not Essential for Viability in *C. albicans*

To study the function of Sod5p in *C. albicans*, we created a strain in which both copies of *SOD5* were deleted. We generated a strain UH16 with the *sod5::HIS1 sod5::hisG-URA3-hisG* constructs replacing both copies of the *SOD5* gene (see MATERIALS AND METHODS for details). The correct position of insertions during these steps was analyzed by Southern blotting (Figure 6A). The absence of the *SOD5* gene was also confirmed with a Southern blot probed with a *SOD5* internal fragment probe. This probe binds inside the *SOD5* open reading frame exclusively, and it fails to generate a *SOD5*-corresponding band in the *SOD5* double-mutant (our unpublished data). When the DNA was restricted with *DraI*, the *SOD5* probe hybridized to an unexpected 7-kb DNA fragment, which could be derived from the accumulation of a 7-kb DNA fragment seen with ethidium bromide staining. The nature of this piece of DNA is unclear, but it might be derived from the multiple repeat



sequence whose *DraI* restriction digest yields a 7-kb DNA fragment that has an affinity to the *SOD5* probe (our unpublished data). In addition, the absence of *SOD5* mRNA under either *SOD5*-inducing conditions (the presence of either serum or alkaline conditions or high pH) or noninducing conditions (YPD) was confirmed by Northern blotting by using the same internal probe (Figure 6B). No increase in *SOD1*, *SOD4*, and *SOD6* transcript levels was observed in

the *sod5* mutant strain (UH16-2) (our unpublished data). Finally, we performed an in-gel activity assay that detects the activity of both types of superoxide dismutases, CuZnSOD and MnSOD. RM1000-HU cells grown in Iscove's modified Dulbecco's medium (IMDM) at 37°C express both MnSOD (Figure 6C, lane 1, top band) and high amounts of CuZnSOD (Figure 6C, lane 1, bottom band). On the other hand, UH16-2 cells showed reduced levels of the CuZnSOD

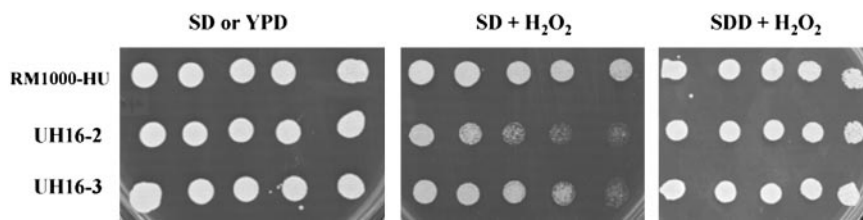


Figure 7. Hydrogen peroxide and limited nutrients inhibit growth of UH16-2 strain. Spot assay of RM1000-HU, UH16-2, UH16-3 strains on SC or YPD, SC supplemented with hydrogen peroxide, and SC supplemented with 2.5% dextrose (SDD) and hydrogen peroxide media at 30°C for 48 h.

activity (Figure 6D, lane 2, bottom band is missing) under the conditions tested. The CuZnSOD activity is restored in the UH16-3-revertant strain (Figure 6C, lane 3, bottom band). The *rim101* mutant strain also showed reduced levels of the CuZn superoxide dismutase activity band (Figure 6C, lane 4). This observation is consistent with the results in Figure 5 and shows that the *RIM101* mutant cells possess low levels of both *SOD5* transcripts and Sod5p enzyme activity. The activity of the CuZnSOD can be inhibited by KCN, which blocked the CuZnSOD activity of RM1000-HU and UH16-3 cells grown in IMDM medium at 37°C (Figure 6C, lanes 5 and 6, bottom bands are missing). This shows that in IMDM media *SOD5* seems to be a major contributor of overall CuZnSOD activity in *C. albicans*. The ability to generate the viable double-mutant strain shows that *SOD5* is not an essential gene in *C. albicans*.

Hydrogen Peroxide Together with Limited Nutrients Impairs Growth of the *SOD5* Mutant Strain

We examined the growth properties of the *sod5* mutant strain under conditions that were shown to increase the transcription of the *SOD5* gene. The spot assay showed that there is no growth difference between the UH16-2 strain and the wild-type strain in medium that contained serum, NaCl, pH 8, or NaCl and pH 8. The *sod5* mutant strain showed no heat sensitivity at 42°C, and no growth defect on Lee and Spider medium (our unpublished data). In addition, the *sod5* mutant strain showed no defect with respect to hyphal development triggered by serum, IMDM media, or high pH (pH 8.0) at 37°C as shown by spot assays and growth in liquid media (our unpublished data).

In *Saccharomyces cerevisiae*, both of the single genes encoding CuZnSOD and MnSOD were found to be up-regulated during the metabolic transition from fermentation to respiration (Maris *et al.*, 2001), and the *S. cerevisiae* CuZnSOD mutant possesses a reduced ability to grow by respiration in glycerol-rich medium (Longo *et al.*, 1996; De Freitas *et al.*, 2000). We therefore investigated the ability of the *sod5* mutant strain UH16-2 to grow on media rich with nonfermentable carbons. We found that there was no growth difference in the liquid or solid medium that contained either glucose, galactose, glycerol, or methanol as a carbon source; in the absence of any of above-mentioned carbon source molecules, the *SOD5* mutant grew as fast as its parental strain (our unpublished data). CuZnSOD deficient *S. cerevisiae* require lysine and methionine for aerobic growth (Bilinski *et al.*, 1985), whereas superoxide dismutase defective *E. coli* require branched chain amino acids (Imlay and Fridovich, 1991). However, the *C. albicans* *SOD5* mutant showed neither lysine, methionine, nor branched amino acid auxotrophies (our unpublished data).

The *E. coli* and in *Helicobacter pylori* *SOD* mutants were found to be more sensitive to the effect of hydrogen peroxide than its parental isogenic strain (Carlioz and Touati, 1986; Keyer *et al.*, 1995; Seyler *et al.*, 2001). We compared the growth rate of the *SOD5* mutant with that of its parental

isogenic strain. We found no growth difference between the *SOD5* mutant and the wild-type strains grown on nutrient-rich media (YPD) supplemented with 1.0 mM hydrogen peroxide (Figure 7). However, the *SOD5* mutant (UH16-2) grew poorly on SC (synthetic complete) media supplemented with 1.0 mM hydrogen peroxide, compared with its parental strain (Figure 7). The growth of UH16-3 strain showed an intermediate growth on SC media supplemented with hydrogen peroxide, consistent with the reintroduction of *SOD5* in one copy into the UH16-1 background. The *SOD5*-phenotype suppression is observed in UH16-2 cells after incubation in SC media, which contains hydrogen peroxide and slight excess of fermentable glucose (2.5% glucose, rather than 2.0% of glucose) or in YPD media which contains hydrogen peroxide (Figure 7).

SOD5 Is Involved in Virulence of *C. albicans* but Is Not Involved in Defense of *C. albicans* against the Fungicidal Activity of Macrophages

The sensitivity to hydrogen peroxide is often linked to a decrease in virulence. To test directly the *SOD5* involvement in macrophage-defense, the UH216-2 strain was incubated with RAW 264.7 mouse macrophage cells, and the percentage of survival ($28.9 \pm 5.7\%$) was not different from the survival of its parental RM1000-HU strain ($27.6 \pm 7.0\%$) and from the revertant UH16-3 strain ($28.9 \pm 7.0\%$). These results suggest that Sod5p is not required for *C. albicans* survival when cells are subjected to the fungicidal activity of macrophages.

To test directly the *SOD5* involvement in an organism defense, we tested the virulence of the *sod5* mutant strain in a mouse systemic infection model by intravenous tail infection (Figure 8). The results showed that 90% of the mice infected with the wild-type (RM1000-HU) died within 8 d of being inoculated. However, the homozygous *sod5* mutant strain (UH16-2) had markedly diminished virulence compared with the wild-type parental strain (RM1000-HU). Mice infected with UH16-3 strain showed an intermediate survival rate consistent with the reintroduction of one copy of *SOD5* gene into the UH16-1 background.

The cfu in mice kidneys were determined on the fifth day after infection. One hundred percent of the kidneys recovered from mice infected with the wild-type parental strain (RM1000-HU) revealed an average of 1.0×10^6 cfu g⁻¹ kidney at day of sacrifice. Seventy-five percent of the kidneys recovered from mice infected with the *sod5* revertant strain (UH16-3) revealed an average of 7.0×10^5 cfu g⁻¹ kidney at the day of sacrifice and 2.0×10^4 cfu g⁻¹ kidney in the remaining 25%. Seventy-five percent of the kidneys recovered from mice infected with the *sod5* strain (UH16-2) revealed an average of 5.0×10^4 cfu g⁻¹ kidney on the day of sacrifice and 5.0×10^5 cfu g⁻¹ kidney of the remaining 25%, indicating an effective clearance of the *sod5* knockout strain. These results demonstrate that a functional Sod5p plays an important role in the virulence of *C. albicans*.

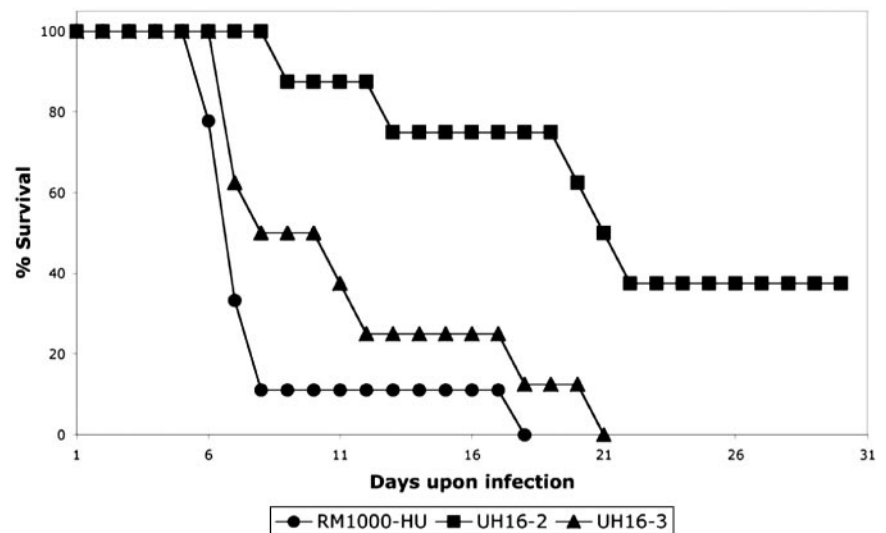


Figure 8. Sod5p activity is required for virulence in animal model. Survival of mice infected with *C. albicans* *sod5* mutant UH16-2 (squares), *sod5* revertant UH16-3 (triangles), and wild-type parental RM1000-HU (circles) strains. Mice were inoculated by tail vein injection and survival was measured over a 30-d period.

DISCUSSION

C. albicans is known to express a CuZnSOD (*SOD1*) and an MnSOD (*SOD3*) in the cytosol and a second MnSOD (*SOD2*) in the mitochondria (Hwang *et al.*, 1999; Rhie *et al.*, 1999; Lamarre *et al.*, 2001). In this study, we report the existence of three additional candidate CuZn-containing superoxide dismutases, the products of the *SOD4*, *SOD5*, and *SOD6* genes. We have characterized the *SOD5* gene that seems to be important factor in the virulence of this fungus. Virulence of *C. albicans* is often attributed to its ability to switch from yeast to hyphal forms. Consistent with this, *SOD5* expression increases with several hyphal-inducing conditions, *SOD5* expression is dependent on known components of hyphal-inducing signaling pathways, and the absence of this gene leads to a defect in the virulence of this organism. As expected for superoxide dismutases, *SOD5* has some roles in responding to certain stresses.

Superoxide dismutases serve to convert damaging superoxide radicals, a key form of ROS, to less damaging hydrogen peroxide that can be converted into water by catalase action. *SOD5* clearly encodes a CuZn-containing superoxide dismutase: it has the highest sequence similarity to the CuZn superoxide dismutase family, and deletion of the *SOD5* gene eliminates the major source of CuZn dependent (cyanide sensitive) superoxide dismutase activity detected in IMDM-grown cells.

What is the significance of *C. albicans* having multiple CuZn superoxide dismutase genes, most of which are potentially expressed in the cytosol? For pathogens such as *C. albicans*, there are both internal and external sources of superoxides. An attractive model would be to have high levels of SOD activity to protect the fungal cell against the oxidative burst inside the phagosome. The fact that *SOD5* is highly expressed in the hyphal state of *C. albicans* (this study; Nantel *et al.*, 2002), and hyphal cells are typically the form that interacts with the host phagocytic cells (reviewed in Gow *et al.*, 2002) would support such a model. In addition, increases in pH, salt concentration, or presence of oxidative species lead to increases in *SOD5* transcription, the same events happen inside the phagosome (Reeves *et al.*, 2002), further strengthening a model that *SOD5* could have a defense-related function. Most significantly, the strongest evidence that supports this model is the fact that the *sod5* knockout strain is defective in mouse killing. It was previ-

ously shown that the *C. albicans* *sod1* mutant strain is defective in virulence and in macrophage killing. Our results have shown that the *sod5* mutant behaves similarly to *sod1* with respect to the virulence in mice, but surprisingly Sod5p does not affect macrophage killing. This would suggest that there are other phagocytic mammalian cells, such as neutrophils or dendritic cells, to which Sod5p is critical for a wild-type response.

We investigated the possibility that a further function of *SOD5* is to protect *C. albicans* cells against intracellularly generated superoxide radicals. *C. albicans* produces the highest levels of CuZnSOD during the midexponential phase (Rhie *et al.*, 1999; Lamarre *et al.*, 2001), and it generates increased amounts of CuZnSOD during the switch from yeast to hyphal forms (Gunasekaran *et al.*, 1998). During the same conditions, hyphal growth and in exponential phase of the growth, *C. albicans* is known to produce more ROS, including superoxide radicals (Schroter *et al.*, 2000). These two parameters, superoxide generation and superoxide detoxification, were correlated to respiration of *C. albicans* (Ito-Kuwa *et al.*, 1999). Mitochondrial respiration was shown to be a powerful source of superoxide radicals in yeast (Chance *et al.*, 1979), and factors such as nonfermentable carbon sources were linked to increased superoxide production by electron transport chain in *C. albicans* (Rikhvanov *et al.*, 2002), *S. cerevisiae* (Davidson *et al.*, 1996; Longo *et al.*, 1996; Maris *et al.*, 2001), and in *Schizosaccharomyces pombe* (Jeong *et al.*, 2001). In our study, these conditions that increase ROS production also increase *SOD5* transcription. *SOD5* expression levels were increased when cells grew in the presence of salt or on nonfermentable carbon as the only carbon source. In addition, an increased amount of fermentable glucose restored the growth ability of the *SOD5* mutant cells grown in nutrient limiting media in the presence of hydrogen peroxide. We also observed that *SOD5* transcription was induced by both menadione and riboflavin, which are intracellular and extracellular superoxide generators, respectively. This also provides us with the evidence that Sod5p is needed for elimination of superoxides found on both sides of the cell membrane. This might suggest that Sod5p shares the redundancy with other superoxide dismutases in eliminating extracellularly and intracellularly generated superoxide radicals in *C. albicans*.

All the increases in *SOD5* transcript levels were observed in *C. albicans* grown in exponential phase, the same phase in which *C. albicans* produces more ROS. All hyphal-inducing conditions induced *SOD5* transcription, and *SOD5* transcription was found to be regulated by transcription factors regulating the yeast-to-hyphal switch of *C. albicans*. All these lines of evidence might suggest that hyphal growth and log-phase growth processes increase mitochondrial respiration in aerobically growing *C. albicans*, which subsequently increase mitochondrially generated superoxide radicals, leading to increased levels of detoxification enzymes. It is possible that these detoxification enzymes include proteins such as *SOD5*.

Our results also show that *SOD5* possesses a complex regulation of expression. Intriguingly, Efg1p was found to positively regulate *SOD5* transcription in the presence of serum as seen in Nantel *et al.*, 2002, and negatively regulate *SOD5* transcription under noninducing conditions. In addition, *SOD5* transcription was found to be dependent on the Rim101 transcription factor, which is required for alkaline-induced filamentation (Davis *et al.*, 2000b). This implicates the Rim101p pathway in the response of the *C. albicans* cell to oxidative stress. Rim101p was shown to be required for *SOD5* induction in response to a variety of external signals, not just pH. Interestingly enough, the growth of the *S. cerevisiae* *rim101* mutant was found to be inhibited at basic pH and in the presence of NaCl (Lamb *et al.*, 2001), the same conditions in which *SOD5* transcription was found to be up-regulated. The 1-kb sequence upstream of the *SOD5* start codon revealed four potential Rim101p binding sites, which closely resemble the previously described CCAAGAAA–Rim101p binding site (Ramon and Fonzi, 2003), and four potential Efg1p binding sites (CAWWTG) (Leng *et al.*, 2001).

All recent evidence suggests that *C. albicans* possesses a complicated cytosolic detoxification system. During the log-phase growth of *C. albicans*, *SOD1* and *SOD5* (CuZn-containing superoxide dismutases) are expressed (this study; Lamarre *et al.*, 2001). *SOD1* seems to be primarily responsible for removal of extracellular, macrophage-generated superoxide radicals (Hwang *et al.*, 2002). *SOD5* seems to be responsible for detoxification of extracellularly and intracellularly, possibly mitochondrially, generated superoxide radicals during log phase and hyphal growth. As *C. albicans* cells enter the stationary phase, the expression of CuZn-containing superoxide dismutases is repressed and the expression of Mn-containing cytosolic superoxide dismutase (*SOD3*) is increased (Lamarre *et al.*, 2001).

The search for sequences similar to CuZnSOD in genomes of *Neurospora crassa*, *Aspergillus fumigatus*, *S. cerevisiae*, and *S. pombe* revealed that all of these organisms have *SOD1* homologs, but only filamentous fungi (*N. crassa* and *A. fumigatus*) have additional sequences homologous to *SOD5* [NCU03013.1 (161682–160794) and contig:69: a *fumigatus* (74890–75198), respectively]. Perhaps filamentous fungi have more sources of production of harmful superoxide radicals, which applies more pressure on these fungi to possess extra isoforms of superoxide detoxifiers, such *SOD5*. The role of *SOD4* and *SOD6* remain to be established, although *SOD4* was recently shown to be regulated in White-Opaque phenotype switching (Lan *et al.*, 2002).

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